#10 MP 1/28/03 w/attch

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	§	
	§	
AVINOAM DUKLER ET AL.	§	
	§	
Serial No.: 09/783,083	§	
	§	
Filed: May 21, 2001	§	Group Art Unit: 1627
	§	
For: COMBINATORIAL COMPLEX CARBOHYDRATE LIBRARIES AND METHODS FOR THE MANUFACTURE AND USES THEREOF	§	
	§	Attorney
	§	Docket: 01/21362
Examiner: Jon D. Epperson	§	

Commissioner of Patents and Trademarks
Washington, D.C. 20231

DECLARATION OF DR. NIR DOTAN UNDER 37 CFR 1.132

I am presently employed as the President and Chief Technology Officer of Glycominds Ltd., the assignee of the present invention. I received my Ph. D degree from Tel-Aviv University, Tel Aviv, Israel, in the field of protein-carbohydrate interactions. I am a co-inventor of the subject matter claimed in the above-referenced U.S. patent application.

I have read the Official action issued with respect to the above-identified application.

The following briefly describes the document enclosed along with this declaration, which documents clearly illustrate that the profiling method of the present invention is enabled by the instant specification as well as being novel and non-obvious with respect to the prior art of record.

Appendix A

Appendix A illustrates the results of a survey conducted by Glycominds Ltd. in which serum samples of 72 normal blood donors were profiled using complex carbohydrate libraries constructed according to the teachings of the present invention.

Appendix B

Appendix B demonstrates the use of a glycosyltransferase enzyme for *in situ* synthesis of complex carbohydrates on a solid support. The enzyme N-Acetyl-β-D-glucoseamine- β 1,4- galactosyltransferase, EC 2.4.1.90, was used to catalyze the transfer of Galactose from UDP-Gal only to terminal β linked GlcNAc on the non reducing end of complex carbohydrate. Here we demonstrate that one type of an enzymatic elongation step can be utilized to generate a diversity of structures on a glass slide in microarray format. Appendix B clearly demonstrates that glycosyltransferase enzymes are highly suitable for synthesis of diverse structures on a single solid support.

Emails 1 and 2

The email correspondence enclosed herewith provides evidence showing that the present invention was revealed to the scientific community in 2001 and further illustrates the great interest shown by the scientific community in the present invention.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United states Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 23 December 2002

Dr. Nir Dotan

Encl.:
CV of Dr. Nir Dotan

09/783,083 Declaration/watch #

CV of Dr. Nir Dotan
12 Margalit St., Shoham, 73142 Israel
Phone +972-(0)8-9181080, Fax +972-(0)8-9181081, email: nirdo@glycominds.com

EXPERIENCE

Glycominds Ltd., Lod, Israel **1999-present**
Co-Founder, President and Chief Technical Officer

- Co-founded and established Glycominds.
- Co-Invented and led the developement of core company technologies.
- Directed and supervised company R&D team.
- Established relationships with key market players.

Israeli Air Forces – Reserve Duty **1990-present**

- Pilot with rank of Major.
- Active duty 1982 - 1990

EDUCATION

Tel Aviv University – Tel Aviv, Israel
Ph.D. in Biotechnology, October 1998
Doctorate Thesis: *Construction of Supramolecular Structures by Specific Cross-linking of Binding Protein Building Blocks*

Tel Aviv University - G.S. Wise Faculty of Life Sciences
B.Sc. in Biology, April 1993

PATENT APPLICATIONS

Dukler A. and Dotan N. *Method and System for an Integrated Glycomics Filter for Drug Candidate Prioritization.* (September 2000) Provisional – US 60/231,525

Dotan N. and Dukler A. *System and Method for Carbohydrate Sequence Presentation, Comparison, and Analysis.* (May 2000) US 09/573,548

Dotan N. and Dukler A. *System and Method for Managing and Analyzing Carbohydrates Sequences.* (May 2000) US 09/573,554

Dukler A. and Dotan N. *Combinatorial Complex Carbohydrate Libraries and Methods for the Manufacture and Use Thereof.* (February 1999) US 09/251,298

PUBLICATIONS

Dotan N., Arad D., Frolow, F. and Freeman, A. (1999) Self-Assembly of a Tetrahedral Lectin into Predesigned Diamondlike Protein Crystals. *ANGE. CHEM.* - INTER. EDIT. **38**(16) pp. 2363-2366.

Dotan N., Cohen N., Kalid O. and Freeman A. In: Nano - Surface Chemistry, M Rosoff. (editor). Supramolecular assemblies made of biological macromolecules. Marcel Dekker Inc., New York. 2002 (pp.461-472).

Banin E. Neuberger Y. Altshuler Y. Halevi A. Inbar O. Dotan N. and Dukler A. (2002) A Noval Liner Code Nomenclature for complex Carbohydrates. Trends in Glycoscience and Glycotechnology Vol. 14 No. 77 pp. 127-137

NATHAN SHARON - CURRICULUM VITAE

Born November 4, 1925, Brisk, Poland. Emigrated to Israel 1934. Married Rachel Itzikson 1948; two daughters, Esther Sharon (1952) and Osnat Bairey (1956).

***Education*:** 1943, Grad. High School, Tel Aviv; 1950, M.Sc. (Biochemistry) Hebrew Univ. Jerusalem; 1953, Ph.D. (Biochemistry) Hebrew Univ. Jerusalem.

Postdoctoral research:: 1956-57, Biochem. Res. Lab., Mass. General Hospital &Harvard Med. School, Boston, MA., (with F.Lipmann); 1957-58, Lab. for Cabohydrate Res., M.G.H. & H.M.S., (with R.W.Jeanloz); 1958, Dept. Biol., Brookhaven National, Upton, New York (with D.E.Koshland Jr.)

Professional employment: 1949-53, Research Assist., Agricultural Res. Station, Dairy Res. Lab., Rehovot ; 1954-, **Dept. of Biophysics, Weizmann Inst. Science**, Rehovot: Res. Assistant, 1954; Res. Associate, 1957; Senior Scientist, 1960; Assoc. Prof.,1965; Prof., 1968; Head, Dept. Biophys., 1973-83, 1987-90; Dean, Faculty of Biophys.-Biochem., 1976- 77, 1980-83, 1984-86; Prof. Emeritus, 1995.

Other appointments: 1962-1963, Res. Assoc., Dept. Biol. Chem., Harvard Med. School; 1963, Visit. Assoc. Prof., Dept. Biochem., Albert Einstein College of Medicine, New York;1968, Visit. Prof., Lab. Molecular Biol., Oxford University, UK; 1968, Visit. Prof., Lab. for Molecular Biol., Oxford University, UK; 1970-1971; Visit. Prof., Dept. of Biochem., Univ. California, Berkeley; 1971, Visit. Prof., Dept. Chemical Pathol., St. Mary's Hospital Med. School, London; 1977-78, Scholar-in-Residence, Fogarty International Center, NIH, Bethesda, MD; 1978, Visit. Prof., Dept. Biological Sciences, Univ. California, Santa Barbara;
1981, Scholar-in-Residence, Fogarty International Center, NIH, Bethesda, MD; 1983-84, Distinguished Visiting Scientist, NIADDK, NIH, Bethesda, MD; 1991, Greenberg Scholar, Oklahoma Medical Research Foundation, Oklahoma City, OK; 1992-1993, Visit. Prof., Harvard Med. School; 1993, Distinguished Visit. Scientist, Roche Inst. Mol. Biol., Nutley, NJ.

Elected positions: 1972-74, 1988-90, Chairman, Scientific Council, Weizmann Inst. Science. 1990- Chairman, Panel of Advisors, Batsheva de Rothschild Foundation for the Advancement of Science in Israel.

Awards and Honors

1973-Landau Prize, Mifal Hapayis
1976-Member, European Molecular Biology Organization
1977-Fogarty International Scholar, NIH, Bethesda, MD.
1977-Weizmann Prize in Exact Sciences, from the City of Tel Aviv
1980-Honorary Member, American Society of Biological Chemists
1987-Visiting Prof., College de France, Paris
1987-Datta Lectureship Award, Federation of European Biochemical Societies
1989-Olitzki Prize, Israel Society for Microbiology
1989-Bijvoet Medal, Utrecht University
1990-Docteur Honoris Causa, Université René Descartes, Paris
1991-Special issue of *Carbohydrate Research* on lectins
1992-Member, Israel Academy of Sciences and Humanities

1994-Israel Prize in Biochemistry and Medicine
1994-Visiting Prof., College de France, Paris
1999-Foreign Member, Academia Europaea
2000-Foreign Member, Polish Academy of Sciences
2001-Foreign Member, American Society fir Microbiology
2001-Honorary Fellow, Open University of Israel

Publications: Over 450 scientific publications, including review articles (e.g., 3 in *Annu. Rev. Biochem.*, 3 in *Science*, 5 in *Scient. American*, etc.) Seven of the publications have been selected as Citation Classics. Listed among 1000 most cited scientists 1965-1978 (Current Contents, Oct. 12, 1990) and ranked 260th among "Citation Superstars 1973-1984" (The Scientist, July 6, 1990)
Books: Three in Hebrew on popular science; also: *Complex Carbohydrates, Their Chemistry, Biosynthesis and Functions*, Addison Wesley Publishing Co. 1975, (Japanese translation by T. Osawa, Tokyo University Press, 1977); *Biotechnological Applications of Proteins and Enzymes* (ed. with Z. Bohak), Acad. Press, NY, 1977; *The Lectins: Properties, Functions and Applications in Biology and Medicine* (ed. with I.E. Liener and I.J. Goldstein), Academic Press, NY.,1986. "Lectins" (with H. Lis), Chapman & Hall, London, 1989. (Japanese translation by T. Osawa and Y. Konami, 1990). (Second edn. in preparation).
Personal recollections: Lectins: from obscurity into the limelight, *Protein Science* 7, 2042-2048 (1998); Half a century between carbohydrates and proteins, *Comprehensive Biochemistry – History of Biochemistry* 41, 391-448 (2000).

Memberships in Scientific Societies: American Chemical Society; The Biochemical Society (UK); International Science Writers Association; Israel Biochemical Society (President 1969-1970); Society for Complex Carbohydrates; Federation of European Biochemical Societies (Chairman 1980-81); International Glycoconjugate Organization (President 1989-91).

Editorial Boards (past and present): *Advanc. Carbohydrate Chem. Biochem.; Acta Biochim. Polonica; Biomed. Chromatography; Biochim. Biophys. Acta; Carbohydrate Res.; Chemtracts-Biochem & Molec. Biol.; Europ. J. Biochem.; Europ. J. Immunol.; FEBS Lett.; Glycobiology; Glycoconjugate J.; J. Molec. Recognition; Molec. Cellular Biochem.; Trends Glycoscience Glycotechnol.*
Guest editor of special Journal issues or sections: Lectins (*Glycoconjugate J.* 1994); Lectins (*Chemtracts* 1996); Glycoconjugates (*Biochimie* 2000); Carbohydrates and glycocnjugates (Current Opin, Struct. Biol. 1998, 2002, 2002).

Email-1 - Chips To Hits

-----Original Message-----
From: Gioia [mailto:gioia@genemaster.com.tw]
Sent: Friday, November 23, 2001 3:46 AM
To: arig@glycominds.com
Subject: abstract request

Dear Dr. Gargir,

Awaring and was impressed by your poster presentation at IBC's Chips to Hit Microarray conference on October 28-Nov 1, 2001, we would appreciate the honor if you may send us your presentation file titled as follows for our further reference and pursuance.

Poster Presentation Title: "GlycoChipT- HighThroughput Technology for Screening & Analysis of Protein- Glycan Interactions"

GeneMaster Lifesciences Co., Ltd. is the leading biochip operator at Taiwan with its proprietary MasterChip and GalaxyChip platform technology. For corporate details, you are welcome to visit our website at: www.genemaster.com.tw.

GeneMaster had the pleasure hosting the 2001 Taipei International Master Forum on Biotechnology with theme on Biochip, Bioinformatics and Biopharmaceuticals on August 27 – 29, 2001, where there were over 24 international biotech elite speakers from across government, academic and industry representatives with extensive media exposure and participation. **GeneMaster is now organizing 2002 Taipei International Master Forum on Biotechnology, scheduled on June 24 – 26, 2002 at Tri-Service General Hospital, Taipei, Taiwan, and would extend our invitation for your participation.**

Master Forum is an annual event targeting to congregate international elites from the biotechnology and pharmaceutical industries to share experiences and to exchange information as the bridging intermediary to expedite industry development. **We welcome any synergistic initiative to make Master Forum the major bridging intermedia1ry internationally for industrial intelligence across government, academia and the industry.**

Master Forum briefing with event picture is available at : http://www.genemaster.com.tw/english/e-events.htm for your reference.

Our business philosophy is to yield value to be exploited by others. Should there be anything whereby we may be at your service, please do not hesitate to let us know. It will be our pleasure to be at your service.

With appreciation, we remain

Sincerely Yours

Howard Wang
Assistant Vice President/Business Development
GeneMaster Lifesciences Co., Ltd.
Tel:886-2-32340930 ext 101
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e-mail: howard@genemaster.com.tw

Email-2 - Protein Microarray Technology lecture

Dear Dr Dotan,
Thank you so much for your reply. I am delighted that you are interested in speaking at the forthcoming Protein Microarray Technologies Conference. I apologise for the short notice, but I am signing off the conference brochure on Friday 1st June. So if possible, I will need you to send your confirmed title and abstract to me by tomorrow afternoon, at the latest, if it is to be included in the brochure. You submitted an abstract for your poster at DDT, so we already have the following on file.

Glycochips - mapping protein interactions with sugars
Dr. Nir Dotan, Founder, President and Chief Scientific Officer, Glycominds, Israel

Using a novel glycomics approach, Glycominds' GlycoChip(tm) can identify novel carbohydrate binding proteins as potential drug targets or therapeutics, and discover unique binding inhibitors by providing rapid and specific analysis of protein to glycan interactions. In conjunction with their vast presence, glyco-molecules play critical roles in a myriad of physiological and pathological reactions ranging from immunity to blood clotting to cell death to cell development. Despite their tremendous functional significance, glycans' importance as biological information molecules has been largely overlooked. Glycominds has developed the powerful GlycoChip technology to capitalize on the unmistakable potential of "glyco-knowledge" in the discovery, prioritisation and development of drugs. The Glycochip will permit researchers to efficiently analyse unprecedented numbers of glycan-protein interactions en masse.

We can use this abstract if you wish, or you could send another, if you have some new developments you wish to speak about. I would appreciate it if you could contact me regarding the abstract. If you want to discuss your presentation or the conference, please do not hesitate to contact me.
Thank you for your participation.
Kind Regards,

Lindsay Aspinwall
Conference Producer

-----Original Message-----
From: Sinclair, Emma
Sent: 31 May 2001 09:10
To: Aspinwall, Lindsay
Subject: FW: Protein Microarray Technology Conference

-----Original Message-----
From: Nir Dotan [mailto:NirDo@glycominds.com]
Sent: 30 May 2001 17:48
To: 'Sinclair, Emma'
Subject: RE: Protein Microarray Technology Conference

Dear Emma,
I will be happy to speak at the 'Protein Microarray Technology'.

I will sand you the full registration form and the abstract by the beginning of next week.

Best regards,

Nir Dotan

-----Original Message-----
From: Sinclair, Emma [mailto:emma.sinclair@informa.com]
Sent: Wednesday, May 30, 2001 3:17 PM
To: 'nirdo@glycominds.com'
Subject: Protein Microarray Technology Conference
Importance: High

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Dear Dr Dotan,

Please find attached an invitation to speak at IBC's forthcoming conference on 'Protein Microarray Technology', which is scheduled in Berlin, Germany from 27th-28th September 2001. The formal invitation and programme are attached for your information. If you should not be able to join us, we would be happy to extend this invitation to a colleague and would appreciate it if you could forward this information to the most appropriate person within your organisation. I look forward to hearing from you soon as to whether you will be able to join us in September.

Kind regards

Lindsay Aspinwall

<<DotanInvite.doc>> <<Preliminary Program3.doc>>

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APPENDIX A

A New Type of Glycan Array and Its Use for Profiling of Anti-Glycan Immunoglobulins in Human Serum - Presence of Novel Commonly Occurring Anti-Glycan Antibodies

Darft #4 20/11/2002

Mikael Schwarz, Larissa Spector, Ari Gargir, Avraham Shtevi, Monica Gortler, Rom Altstock, Avinoam Dukler and Nir Dotan
Glycominds Ltd. 1 Yodfat St., Alon Bldg., Lod 71291 Israel

Abstract. **A new type of glycan array based on covalently linked mono- and oligosaccharides is described. The glycans are bound to the surface through a long linker via their reducing ends and are therefore presented to the medium with the same directionality as in nature. They are thus accessible for specific binding by glycan binding proteins such as antibodies, lectins and glycosyltransferases. Using this array we have analyzed the glycan binding antibody repertoire in a healthy human population and detected some previously known anti-glycan antibodies and antibodies against monosaccharides that are constituents of commonly occurring bacterial polysaccharides. While there are general similarities amongst individual anti-glycan binding profiles, each individual had a distinctive profile. A novel anti-cello-oligosaccharide antibody was discovered, which was found to bind to crystalline and amorphous cellulose as well as oligosaccharides of N-acetylglucoseamine, but not to α-linked pyranosides. It is therefore suggested that the anti-cellulose antibody recognizes the β4 backbone of glucose and N-acetylglucoseamine polymers.**

Introduction

Glycans, due to the large number of saccharide building blocks and the variety of linkages between them have an enormous potential to carry information – far exceeding that of nucleic acids or proteins. These glycans are displayed on the surface of macromolecules and cells where the information they encode is deciphered by glycan binding proteins in numerous processes such as the antigen

recognition machinery, bacterial and viral adhesion to host cells and evasion from host immune system, and protein folding, stability and trafficking.

The same attributes that confer the huge information-carrying capacity of the glycans make their synthesis difficult. Likewise, the analysis of protein-glycan interactions suffers from tedious methodology such as erythrocyte agglutination, equilibrium dialysis, affinity chromatography etc. Thus, although much information has become available on the role of glycans in biological systems, progress has been hampered due to difficulties in synthesizing and analyzing these molecules. Much effort has therefore been invested in the development of glycan arrays - analogous to the gene and protein array paradigm – and has yielded a number of methods for binding glycans to solid supports (3,13).

Here we report on a novel glycan array containing chemically defined mono- and oligosaccharides representing naturally occurring glycoconjugates. We also report on its use for the analysis of circulating anti-glycan antibodies in a healthy human population. The array exhibits robust reproducibility and facilitates rapid and convenient analysis of multiple serum samples on large numbers of glycans. We found high levels of antibodies against monosaccharides that are constituents of commonly occurring bacterial polysaccharides like N-acetylglucosamine (GlcNAc) and rhamnose (Rha); antibodies against the Galili epitop (Galα1-3Galβ1-4GalβGlcNAc); and antibodies against β1-4Glucose oligomers. These antibodies were affinity purified and further characterized using glycan arrays as well as other conventional techniques.

Results

The Glycan Array Characteristics. The glycan array presents covalently attached mono- and oligo-saccharides (Fig. 1A) in a microtiter plate format that allows for specific recognition and binding of glycan binding proteins. In the experiments described here, 35 different glycans were immobilized as listed (with linear code

abbreviations) in Table. 1. As can be clearly seen, using this array, lectins bound specifically to their respective ligands (Fig. 1B) – and binding was inhibited by their appropriate soluble saccharides as exemplified by the inhibition of Erythrina corallodendron (ECorA) by Gal (β 1-4) GlcNAc (β) (Fig. 1C). As can be seen from the difference in signal intensity obtained from concanavalin A (ConA) binding to α-Man vs. β-Man and Bandeiraeia simplicifolia (BSI) binding to α-Gal vs. β-Gal and α-GalNAc vs. β-GalNAc, the strength of the binding is influenced by the anomeric configuration of the glycosidic bond to the linker (Fig. 1D). Signal-to-noise ratios were around 10-20 for positive control wells.

Reproducibility. The median coefficient of variation of the quadruplicate samples was found to be between 5-20% (not shown). Using well characterized lectin-glycan couples (e.g. wheat germ agglutinin (WGA) and β-GlcNac), the plate-to-plate coefficient of variation was determined to range between 2% and 10 % (1D). Additionally, three batches of glycan arrays, prepared separately on three different weeks, were analyzed simultaneously to determine batch-to-batch variability. As can be seen in Figure (1A), excellent reproducibility was observed between the different batches as measured with biotinylated WGA and Europium conjugated Streptavidin; βGlcNAc-containing glycans gave rise to signals with a coefficient of variation of 16% between the batches. Day-to-day variation was also monitored by measuring the levels of serum anti-glycan antibodies in a healthy population. In Figure 1A (insert) average population signal is depicted for each glycan in two separate experiments. The correlation coefficient (R2) was 0.95, and a t-test showed there was no statistically significant difference between experiments performed on different days ($\alpha=0.05$; $p>0.1$). Comparison of signals obtained from individual sera in the two different experiments, yielded similar correlations (not shown).

Anti-glycan antibodies in a Normal Human Population. Total Ig antibody binding (as measured with Protein A) of the 72 individual sera against 35 mono- and oligosaccharides is shown in Figure 2A. Table 1 notes those oligosaccharides

of which supported significant levels of antibodies binding. The strongest signals were recorded for antibodies against α-GlcNAc and α-L-Rha, while lower levels were observed against β4-linked oligosaccharides of glucose, α-Gal and Gal(α1-3) Gal. Examination of the antibodies present in an IgG pool gave results similar to those observed for the average of individual sera with high levels of anti- α-GlcNAc, anti α-L-Rha, and anti- β4 linked glucose oligosaccharides (Fig. 4A). It is evident that considerable variation in anti-glycan antibody levels exists between individuals within this normal population. The individual antigenicity profiles of all sera tested on the glycan array are depicted graphically in Figure 2B, in which the strength of the signal is represented by shade of color. It is apparent that no two individuals exhibit identical profiles, but also that there seems to exist considerable similarity between the profiles of many sera such that clusters of these profiles are apparent.

Specificity of Anti-Glycan Antibodies. Binding of serum to immobilized α-GlcNAc and α-L-Rha was inhibited by the corresponding p-nitrophenyl derivative, with a maximal inhibition of 80-100% at about 50μM and with an IC50 of 10μM (Figure 3). In contrast, neither p-nitrophenyl glycerol (Figure 3) or other non-relevant p-nitrophenyl glycosides (not shown) had any effect on the binding. This confirms that binding of the antibodies to the array is glycan specific and not simply adsorption to the well surface.

In order to further characterize the glycan binding antibodies, we affinity purified some of them from the IgG pool by affinity chromatography on Sepharose bound pNP-αGlcNAc, pNP-αRha, pNP-cellotriose and pNP-di-N-acetylchitobiose. The affinity- purified antibodies against α-GlcNAc and α-L-Rha were highly specific to their respective antigens and bound significantly only to saccharides containing the monosaccharide against which they were purified. The anti-cellotriose antibody, however, recognized not only the cello-oligos on the array, but bound significantly also to Lactose and di-N-acetylchitobiose (Figure 4). Binding studies with crystalline and amorphous cellulose show that the anti-cellotriose antibody binds

not only to β4 glucose oligomers, but also to crystalline and amorphous cellulose (Fig 5). Antibodies purified on pNP- di-N-acetylchitobiose beads exhibited a different glycan binding profile than the anti-cellotriose antibody, binding primarily to β-GlcNAc containing glycans and to a smaller extent also to the β1,4 linked glucose oligomers.

Discussion.

The field of Glycobiology is following in the footsteps of Genomics and Proteomics in the development of tools for high-throughput detection and characterization of carbohydrate binding proteins. Some arrays described to date rely on adsorption of glycan or glycan-conjugates to a nitrocellulose solid surface (Wang et al., Fukui et al.,), while another covalently links activated sugars to self assembled monolayers (Houseman and Mrksich). In this paper we report on a novel glycan array and on findings regarding human anti-glycan antibodies in the healthy population obtained by its use. This array displays a number of important features: a) the glycan array utilized simple and well characterized chemistry to covalently link the glycans to the solid support; b) a linker separates the glycan and the solid surface that conferring access to glycan binding proteins and cells, and displaying the glycan to the solution in a defined way which mimics the directionality of the glycans in nature; c) the glycans are chemically defined and are bound to the linker by known anomeric bonds; d) the array is produced in large batches with good well-to-well, plate-to-plate, and batch-to-batch reproducibility and gives excellent signal-to-noise ratios; e) the array format fits standard laboratory equipment like plate-washers and readers; f) the chemistry employed to covalently link glycans to the solid support can readily be adapted to be used on different kinds of material such as plastic and glass; g) pNP-saccharides can be used as acceptors in enzymatic reactions to form complex glycans either on the glycan chip or in solution prior to chemical attachment to the chip surface via the pNP-moiety.

The glycan array described here is well suited for screening of large numbers of specimens such as sera. It is already known that human sera from healthy individuals contain anti-glycan antibodies directed against a variety of antigens like blood groups ABO and Galα1-3Gal that appears on porcine endothelial cells and induces xenograft rejection. Additionally, antibodies against various bacterial glycans including those from Clostridia (18, 19) Klebsiella (14, 15), E.coli (16, 17), Salmonella (13), and Campylobacter (20) have been described. Infection with the latter is sometimes followed by appearance of autoantibodies against certain gangliosides that are associated with MS (20) and Guillian-Barré and Miller-Fisher syndromes (20).

In this work we have expanded the number of mono- and oligosaccharides used for the analysis of anti-glycan antibodies and we have demonstrated the existence of individual profiles of anti-glycan antibodies in the normal healthy population (Figure 3). The demonstration that the technique used here is sensitive and reproducible enough to discern subtle differences between individuals sets the stage for the quest for specific anti-glycan antibody profiles to be used as biomarkers in disease or in prognostic detection of non-responders to drugs, for example. Of course, the profiles obtained in this work are mere snapshots of the anti-glycan antibody status of the studied individuals at a certain time point, and experiments to determine temporal changes in the profiles of a normal healthy population is required. These are currently underway.

In the healthy population studied here we found high levels of antibodies against α-GlcNAc, α-L-Rha and β1,4 linked glucose oligomers. The presence of antibodies against the former two is not surprising since this has been reported in the past (ref) and since these monosaccharides are common components of microbial cell surfaces (7,8,9). We characterized the antibodies by competitive inhibition studies and affinity purification. For example, the anti-α-Rha antibody binding to α-Rha could be inhibited by pNP-α-Rha with IC50 values in the micromolar range, but not by pNP-Glycerol or pNP-β-GlcNAc. In addition,

microarray binding studies using affinity purified anti-α-Rha and anti-α-GlcNAc antibodies showed high specificity when all 35 immobilized glycans were tested. Unlike antibodies to α-L-Rha and α-GlcNAc, high levels of antibodies against β1,4 linked glucose oligomers have not previously been described. Although these structures are only reported to exist in a limited number of bacteria (10,11,12), it forms the most common natural polymer in nature, so the exposure of the immune system to these molecules may be inevitable. Affinity purified anti-cellotriose antibody bound to crystalline and amorphous cellulose and showed specific binding to saccharides linked by β4 bonds, but not by α4, α3, β3 or β6 bonds. This may indicate that this antibody recognizes the β4-backbone of the cellulose chain. Conversely, the purified anti-di-N acetylchitobiose antibody bound specifically to all GlcNAc containing saccharides, but also to β-linked glucose oligosaccharides, which may suggest that the anti-di-N acetylchitobiose antibody prefers the N-acetyl group but also recognizes the β4-glucose backbone. This is in contrast the anti-αGlcNAc antibody which binds solely to the N-acetyl moiety. It is interesting to note that the antibodies described here are naturally occurring antibodies obtained – not by repeated immunization and selective enrichment of relatively few B-cell clones to produce a rather defined polyclonal antibody population – but by pooling of human serum. This may explain the rather low specificity of some of the antibodies examined and makes the high specificity of some of them quite remarkable.

In summary, we have used the glycan array to characterize the glycan binding antibodies in a normal healthy human population, and revealed the existence of several novel abundant antibodies as well as distinct glycan binding antibody profiles in individuals. With these findings as a foundation, we can now continue towards exciting developments in the field of serum glycobiology that will lead to improved and unique capabilities in target discovery, and more notably, identification of novel biomarkers and diagnostics.

Experimental Protocol

Glycan Array. All serum samples were tested using GlycoChip™ (Glycominds Ltd., Lod, Israel). The glycans were covalently bound to the plastic surface through a linker as described in Figure 1A. The array prepared as described (4). A list describing the mono- and oligosaccharides present in the array is provided in Table 1.

Screening of sera. Blood samples were obtained from 72 healthy blood donors under an informed consent protocol approved by the Helsinki Human Studies Ethical committee of the Belinson Medical Center in Tel- Aviv, Israel. The blood samples were collected in evacuated silicon coated tubes containing gel for easy separation of sera from clot (Estar Technologies Cat# 616603GLV). After coagulation of the blood, serum was separated by centrifugation, collected and kept frozen at –25°C until use.

Eighteen glycan array plates containing two different glycans each were used to analyze 36 serum samples in quadruplicates per experiment. Therefore, two days were required to screen all 72 sera on 35 glycans. In addition to the sera samples, a pool of purified IgG (diluted 1:20 in 50mM Tris buffer pH 7.5, 0.15M NaCl, 0.1 % Tween 20 (TBST)) collected from some 10,000 persons (kindly provided by Dr. Israel Nur, Omrix Ltd., Rehovot, Israel) was applied to the array. All samples were tested twice on every glycan.

The volume of all solutions dispensed to the glycan array plate was 10µl/well. The sera were diluted (1:20) in TBST, dispensed into glycan array plates using a Tecan Genesis Workstation 200 robot, and incubated for 30min at 25°C.

The plates were then washed with 250 µL/well of high salt buffer (0.15M KNa pH 7.2, NaCl 2M, MgSO4 0.085M, 0.05% Tween 20) in an automatic plate washer (Tecan, PowerWasher™). At this point, depending on the antibody class measured, the following reagents were added: for total Ig determination: biotinylated Protein A (ICN Biomedicals, Cat No 62-265); for IgG determination biotinylated goat anti- human IgG (Fcγ, biotin-SP-conj, Jackson, Cat. No. 109-065-008); and for IgM measurement, biotinylated goat anti- human IgM (Fc5µ, biotin-SP-conj.,

Jackson, Cat. No. 109-065-043). All three reagents were dispensed manually at 1μg/ml. The plate was then incubated for 30 min at 25°C. At the end of the incubation period, the plates were washed with high salt buffer and Streptavidin-conjugated europium (0.1μg/ml; Wallac, Cat. No. AD0062), was added manually to each well followed by incubation for 30 min at 25°C in the dark, and washing with high salt buffer. Delfia™ enhancement buffer (Wallac, Cat. No. 730232) was added to the wells and the plates were incubated for 30 to 45 min in the dark. The fluorescence of the wells was read with a Victor 1420 (Wallac) plate reader using time resolved fluorescence settings of Emission at 612 nm and Excitation at 340 nm.

An identical control area with α-Gal, α-Man, β-GlcNAc, biotin and glycerol was dedicated on each plate. These wells were assayed with biotinylated ConA (1.5μg/ml) WGA (15μg/ml), and Bandeiraeia simplicifolia (BSI;15μg/ml), and detected with Streptavidin conjugated Europium (see above). The signals obtained from the different plates were compared to monitor plate-to-plate and batch-to-batch variations.

Inhibition of serum antibody binding to glycans. Serum was preincubated with p-nitrophenyl-α GlcNAc (pNPαGlcNAc) , p-Nitrophenyl-L-Rha (□) (pNPαRha), or p-Nitrophenyl glycerol at various concentrations for 1 hour at 25°C. Aliquots were dispensed into glycan array wells coated with either of the three above mentioned glycans, and incubated at 25°C for 30min The bound antibodies were detected as described above.

Affinity purification of anti-glycan antibodies. Sepharose- pNP-α-GlcNAc, pNP-α-L-Rha, pNP-β-GlcNAc1-4β-GlcNAc, and pNP-cellotriose were prepared as described (5). One ml of 0.3M NaCl, 0.2M Tris pH 7.8, 1% Tween 20 and 0.55ml of 4M NaCl was added to 10 ml of the IgG pool and the resulting solution was incubated with 1ml resin in a 15ml tube for 16hr at 4°C with gentle agitation. The resin was packed in a column and washed extensively with 0.3M NaCl, 20mM Tris pH 7.8, and 0.1% Tween 20 until the absorbance at 280 nm of the flow

through was below 0.02 O.D. The bound antibodies were eluted in 1ml batches with 0.2M glycine buffer at pH 2.8 into tubes containing 50µl 1M Tris-HCl, pH 8.6. The pooled fractions were dialyzed over-night against PBS containing 0.02% Sodium Azide. The purified antibodies were used on the glycan array at saturating concentration determined in preliminary experiments (not shown), except in competitive inhibition studies were a dilution giving approximately 2/3 of maximal signal was used.

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Table 1. Saccharides displayed on glycan array and level of anti-glycan antibody in healthy human population.

Glycan No.	**IUPAC**	Relative Ab Level[a]
1	Gal (α)	10
2	Gal (β)	14
3	Gal (β 1-3) GalNAc (α)	
3	Gal (β 1-3) GalNAc (α)	
4	Gal (β 1-3) GlcNAc (β)	
5	Gal (β 1-4) Glc (β)	
6	Gal (β 1-6) Gal (β)	11
7	GalNAc (α)	
8	GalNAc (β)	
10	Fuc (α)	
11	Fuc (β)	
12	Glc (α)	
13	Glc (α 1-4) Glc (α)	
14	Glc (α 1-4) Glc (β)	13
15	Glc (β)	
16	Glc (β 1-4) Glc (β)	9
17	Glc (β 1-4) Glc (β 1-4) Glc (β)	7
18	Glc (β 1-4) Glc (β 1-4) Glc (β 1-4) Glc (β 1-4) Glc (β	5
19	Glycerol	
20	GlcNAc (α)	1
21	GlcNAc (β)	4
22	GlcNAc (β 1-3) GalNAc (α)	6
23	GlcNAc (β 1-4) GalNAc (β)	3
24	L-Rha (α)	2
25	GalA (β)	
26	Man (α)	
27	Man (β)	
28	Neu5Ac (α)	
29	L-Araf (α)	
30	GlcA (β)	
31	X(α)	
32	X(α)	
33	Gal (β1-3) [GlcNAc (β1-6)] GalNAc (α)	
34	Gal (β 1-4) GlcNAc (α)	12
36	Gal (β1-3) [GlcNAc (β1-6)] GalNAc (α)	8

[a]The average level of antibody in a healthy human population relative to other glycans presented on the array; 1 represents the highest levels of antibody binding. Above 14, binding signal is not above background.

Figures Legend

Figure 1. The glycan array; Chemical structure, specificity of Lectin interaction and reproducibility. (A) The pNP-saccharide is covalently linked at its reducing end to the solid surface via a 1,8-Diamino-3,6-dioxaoctane linker. (B) Batch-to-batch reproducibility of binding of biotinylated WGA to the glycan array. Three batches of arrays were produced and assayed with biotinylated WGA on three separate occasions. (B-Inset) Batch-to-batch reproducibility of binding of serum to glycan array. Correlation of average population binding signal on array saccharides obtained in two consecutive experiments. (C) Specificity of Lectin binding to array. Inhibition of binding of biotinylated EcoRA (15μg/ml) to bound Lactosamine by increasing concentrations of soluble Lactosamine. The lectin was incubated with the lactosamine for 1hr, incubated on the glycan array chip an additional hour and detected with Streptavidin conjugated to Europium. (D) Plate-to-Plate reproducibility of the glycan array. Five identical plates presenting β-GlcNac were probed with biotinylated WGA.

Figure 2. Glycan binding profile of a healthy human population. (A) Anti-carbohydrate antibody binding to assorted glycans in serum samples from 72 individuals as measured with biotinylated Protein A. Each dot represent the average of two experiments, each done in quadriplicate. The box includes signals of 50% of the population. The red and black lines in the box represent the mean and median values, respectively. The boundary of the box closest to zero indicates the 25th percentile, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 90th and 10th percentiles. For each of the sera tested the background levels were set arbitrarily and were subtracted from the absolute value. The average background was $3X10^5$ RFU. (B) Signals of individual sera against a series of glycans. The anti-glycan antibody levels measured in relative fluorescence units (RFU) were transformed using a histogram equalization like method which employs a monotonic, non-linear mapping. This way, the RFU values were re-assigned to range between 0 (blue) and 255 (red). The data were clustered using a simulated annealing algorithm.

Figure 3. Specificity of serum antibodies to glycan array. Inhibition of binding of serum Igs to α-L-Rhamnopyranoside immobilized to the well surface as a function of *p*-Nitrophenyl-α-L-Rhamnopyranoside or *p*-Nitrophenylglycerol concentrations. Amount of antibody bound was measured using biotinylated Goat Anti-human IgG antibody.

Figure 4. Binding profile of affinity purified (A) anti-αRha (B) anti-αGlcNAc and anti-di-N-acetylchitobiose and (C) anti-cellotriose and anti-di-N-acetylchitobiose antibodies to an array of 35 glycans. Amount of antibody bound was measured using biotinylated Goat Anti-human IgG antibody.

Figure 5. Binding of anti-cellotriose and anti-Rha to it's relevant saccharide after incubation with crystalline cellulose. Amount of antibody bound was measured using biotinylated Goat Anti-human IgG antibody.

Figure 1

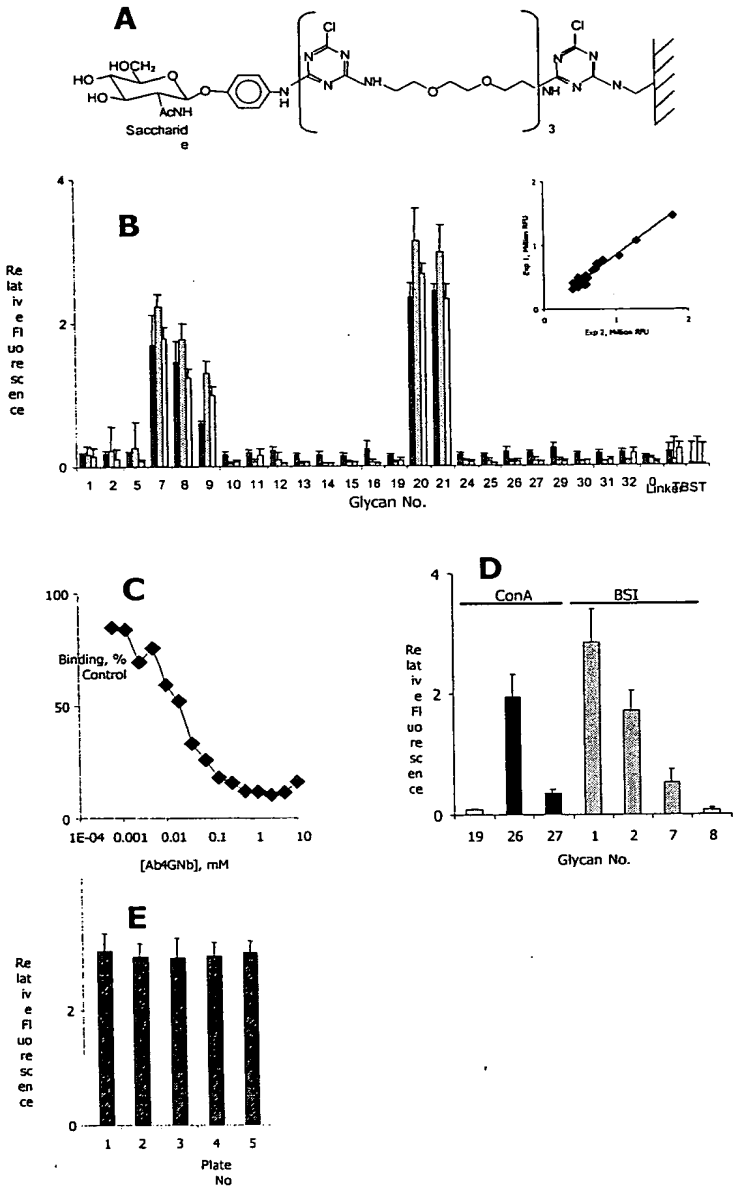

A
Saccharide
3
B
Relative Fluorescence
Glycan No.
Linker
BST
C
Binding, % Control
[Ab4GNb], mM
D
ConA
BSI
Relative Fluorescence
Glycan No.
E
Relative Fluorescence
Plate No

Figure 2

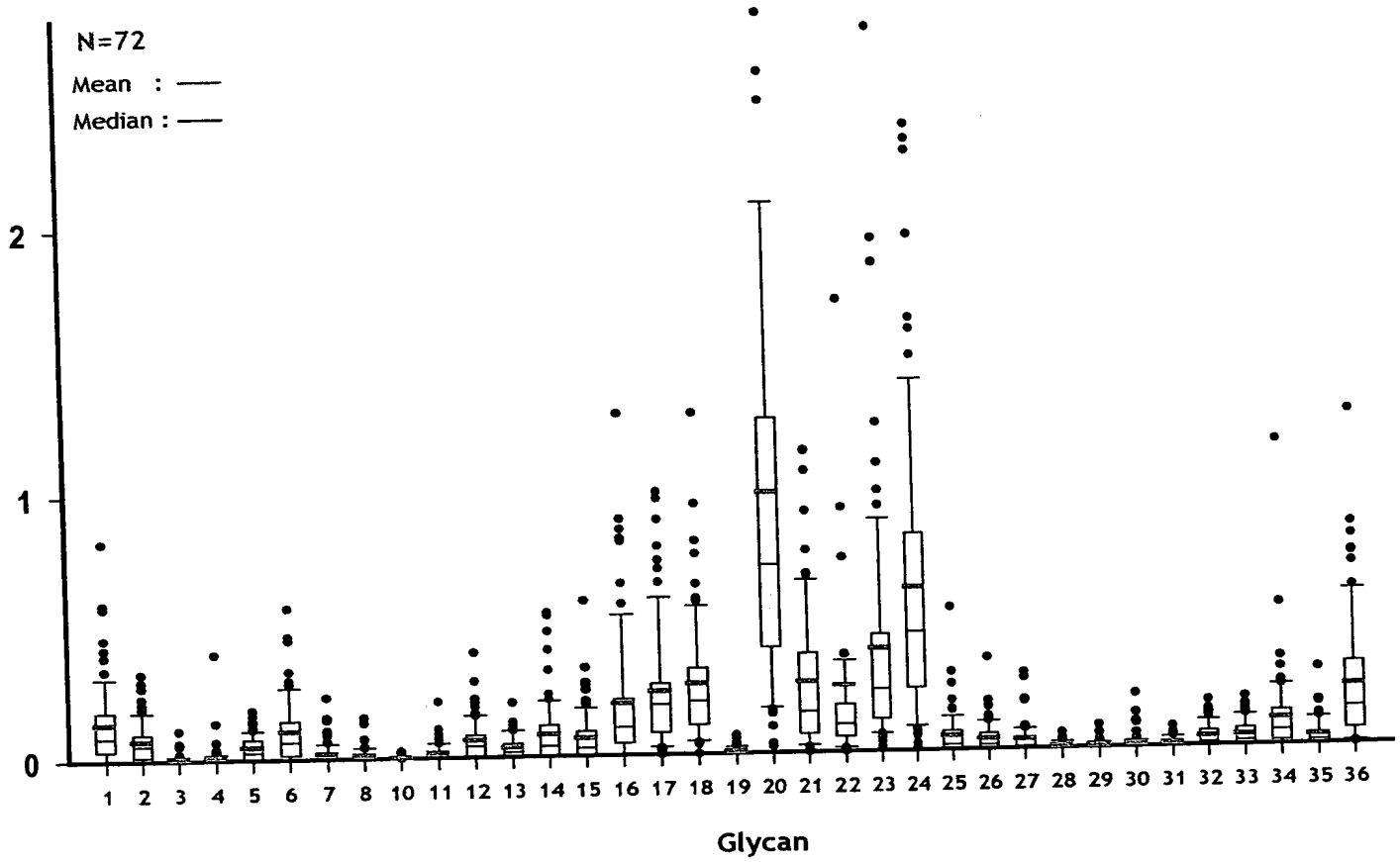

N=72
Mean : ——
Median : ——
2
1
0
1 2 3 4 5 6 7 8 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36
Glycan

Figure 2B.

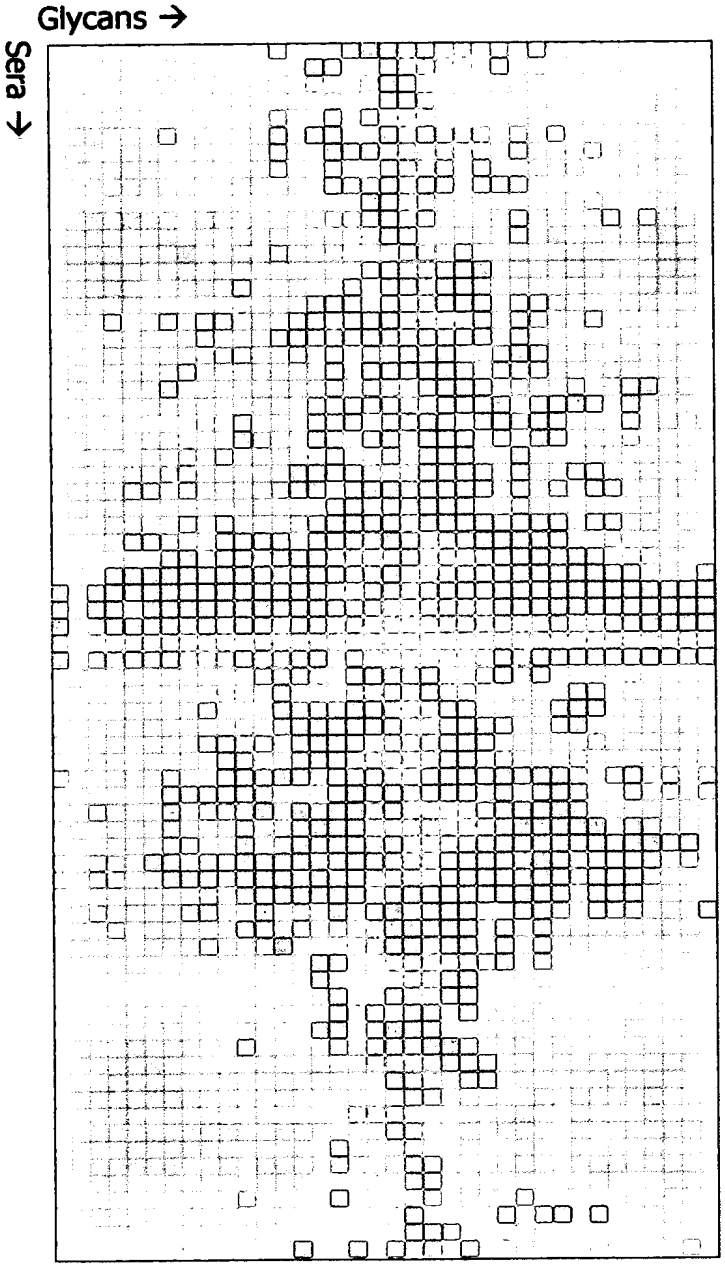


Figure 3

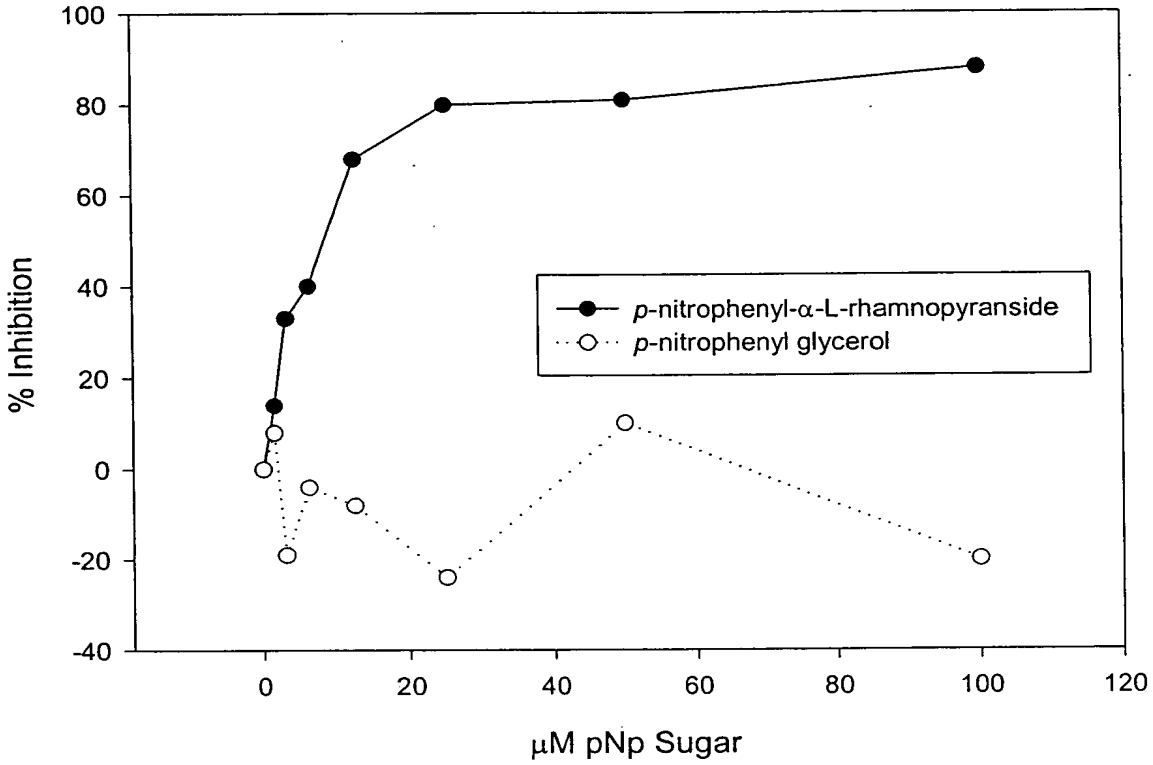
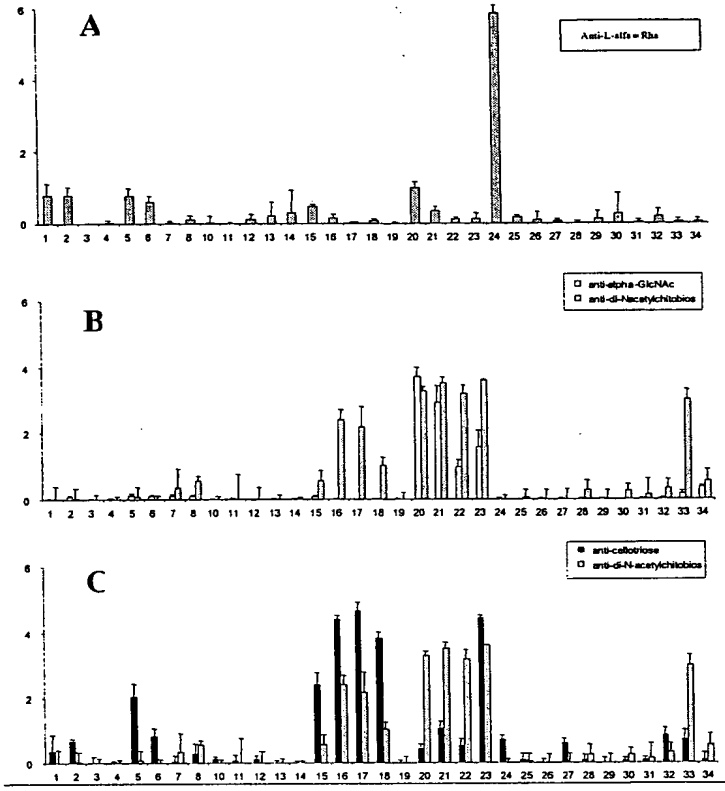
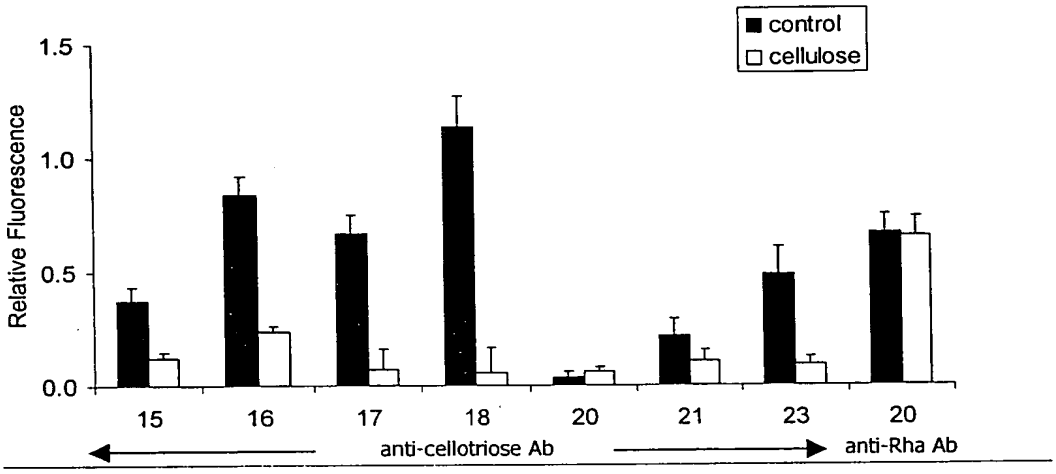

100
80
60
40
20
0
-20
-40
% Inhibition
0
20
40
60
80
100
120
μM pNp Sugar
p-nitrophenyl-α-L-rhamnopyranside
p-nitrophenyl glycerol

Figure 4



Glycan No.	IUPAC
1	Gal (α)
2	Gal (β)
3	Gal (β 1-3) GalNAc (α)
3	Gal (β 1-3) GalNAc (α)
4	Gal (β 1-3) GlcNAc (β)
5	Gal (β 1-4) Glc (β)
6	Gal (β 1-6) Gal (β)
7	GalNAc (α)
8	GalNAc (β)
10	Fuc (α)
11	Fuc (β)
12	Glc (α)
13	Glc (α 1-4) Glc (α)
14	Glc (α 1-4) Glc (β)
15	Glc (β)
16	Glc (β 1-4) Glc (β)
17	Glc (β 1-4) Glc (β 1-4) Glc (β)
18	Glc (β 1-4) Glc (β 1-4) Glc (β 1-4) Glc
19	Glycerol
20	GlcNAc (α)
21	GlcNAc (β)
22	GlcNAc (β 1-3) GalNAc (α)
23	GlcNAc (β 1-4) GalNAc (β)
24	L-Rha (α)
25	GalA (β)
26	Man (α)
27	Man (β)
28	Neu5Ac (α)
29	L-Araf (α)
30	GlcA (β)
31	X(α)
32	X(α)
33	Gal (β1-3) [GlcNAc (β1-6)] GalNAc (α)
34	Gal (β 1-4) GlcNAc (α)
36	Gal (β1-3) [GlcNAc (β1-6)] GalNAc (α)

Fig 5


control
cellulose
Relative Fluorescence
1.5
1.0
0.5
0.0
15
16
17
18
20
21
23
20
anti-cellotriose Ab
anti-Rha Ab

APPENDIX B

Introduction

The following exampled demonstrate the use of glycosyltransferase enzyme for in situ synthesis on a solids phase to create diversity of carbohydrate structures on a single solid phase. The enzyme N-Acetyl-β-D-glucoseamine- β 1,4-galactosyltransferase EC 2.4.1.90 catalyze the transfer of Galactose from UDP-Gal to terminal β linked GlcNAc on the non reducing end of complex carbohydrate. Here we demonstrate one enzymatic elongation step to a set of carbohydrate covalently attached to the solid support creating diversity of natural and non natural structures on a glass slide in microarray format. For monitoring the reaction progression we have used radioactively labeled sugar donor, ^{14}C labeled UDP-Gal. This enable to monitor the reaction by following the amount of C^{14} radioactivity incorporated to the slide surface.

Material and methods

Glass slide substrate: Glass slide printed with Teflon pattern to create 192 flat wells coated with amino propyl silane (Tekdon Inc.) The wells diameter is 1.5mm and the distance between well centers is 2.5 mm. See Figure 1 .

A linker was synthesized on the glass by 3 cycles of activation with cyanuric chloride and elongation as describe in the following.

Cyanuric chloride activation: A solution containing 48 mg of cyanuric chloride (Aldrich, Cat. No. C95501) dissolved in 3 ml of acetone was added, while stirring, to 45 ml of 0.1 M phosphate buffer. Immediately the slides were dipped in the solution for 5 minutes , following which the solution was discarded and the slides washed by dipping three times in double distilled water, and dried by centrifugation

Amino linker elongation cycle: The slides were dipped at 25 °C for 6 hours in 10 % 1,8-diamino 3,6 dioxaoctane (MERCK 818116) solution in water. Following incubation, the slide were washed four times by dipping in water. and dry and dried by centrifugation before coupling of the p-Nitrophenyl-Sugar derivatives.

Different p-Nitrophenyl-Sugar derivative was attached to each wells as follows:

Coupling of p-Nitrophenyl-Sugar derivative: A coupling buffer, 200 mg sodium dithionite and 1.2 ml of 1 M of Na2CO3 buffer, pH 9.6, was added to 40 ml of double distilled water was prepared. 5 µl of a 100 mM solution of each p-Nitrophenyl-Sugar derivative (see Table) in DMSO were added to 1 ml of coupling buffer. 1.3µl drops of each PNP derivative sugar, containing solution was pipette to respective wells immediately after activation, according to the distribution scheme described in Table 1 below. The pipeting was done using a Genensis 200 robotic liquid handling station by Teacan Switzerland. The slide was incubated in a closed chamber to prevent evaporation for 16 hours at 20 °C. The slides were then washed twice with double distilled water and once with methanol. The slides were then left to dry at room temperature for 1 hour and were kept at 4 °C until used. Figure 2 described the final linker with the attached sugar derivatives.

The slide were exposed to enzymatic reaction mixtures

β1,4 Galactosyltransferase reaction: 1.3 µl of 50 mM MOPS pH 7.4 (SIGMA M-9027), 0.2 % Triton CF 32 (Sigma), 20 mM MnCl2 (SIGMA M-9522), 0.5 mg/ml ^{14}C labled UDP-Gal , specific activity 10µCi/mg (Amersham Pharmcia), and 20 milliunits/ml of a recombinant β1,4-galactosyltransferase (Calbiochem 345650) was pipted to the indicated well (columns 1-3). The same mixture without the enzyme was pipeted to the indicated wells (columns 4-8) , see table 2. The slide was incubated at 37°C for 2 hours in a custom-made slide holder, covered with a plastic case to prevent evaporation followed by extensive washing. The slides were exposed to 3 reaction cycles.

After each reaction cycle the slides was exposed to a phosphor imager screen cyclon for 24 hours. A reference slides with known ^{14}C amount in each spot (American Radio Chemicals Inc.) was exposed to the phosphor imager screen as a reference, the radioactivity incorporated to each spot was measured .

TABLE 1

Distribution Scheme of PNP derivative Sugars on the slide before enzymatic reactions

Rows	pNp sugar	Manufacturer and Cat No.
1,2	GlcNAc β	Sigma N-9376
3,4	GlcNAc β 1, 3 GalNAc α	Sigma N-2766
5,6	GlcNAc β 1, 4 GlcNAc β	Sigma N-6133
7,8	Rha α	Sigma N-3641
9,10	Xyl α	Sigma N-1895
11,12	Xyl β	Sigma N-2132
13,14	Gal β 1, 3 (GlcNAc β 1, 6) GalNAc α	TRC N499000
15,16	Gal β 1, 3 GalNAc α	TRC N501255
17,18	GlcNAc β 1, 6 GalNAc α	TRC N499100
19,20	GlcA α	Sigma N-7763
21,22	Fuc α	Sigma N-3628
23,24	Fuc β	Sigma N-2505

Result and discussion

Figure 3 display the radioactivity that was incorporated to the slide after the enzymatic reaction.

The incorporation of radioactivity to each wells with terminal β linked GlcNAc indicate the transfer of Galactose to the terminal β GlcNAc. A similar amount of radioactivity was incorporated to each of the sugar spots . The amount of radioactivity that was incorporated to each of the sugar spots did not changed significantly after repeated reaction cycles (data not shown). There was no

incorporation of radioactivity to the wells that was exposed to reaction mixture without enzyme , and there were no incorporation to carbohydrate with no terminal GlcNAc . The radio graph of the slide is shown in figure 4. The Glycan structures on the slide following the enzymatic elongation are as described in Table 2 .

TABLE 2

Carbohydrate on the slide after the enzymatic elongation step(the bold leters mark the galactose unit that was incorporated)

Rows	Columns 1-3	Columns 4-8
1,2	GlcNAc β	**Gal β 1,4** GlcNAc β
3,4	GlcNAc β 1, 3 GalNAc α	**Gal β 1,4** GlcNAc β 1, 3 GalNAc α
5,6	GlcNAc β 1, 4 GlcNAc β	**Gal β 1,4** GlcNAc β 1, 4 GlcNAc β
7,8	Rha α	Rha α
9,10	Xyl α	Xyl α
11,12	Xyl β	Xyl β
13,14	Gal β 1, 3 (GlcNAc β 1, 6) GalNAc α	Gal β 1, 3 (**Gal β 1,4** GlcNAc β 1, 6) GalNAc α
15,16	Gal β 1, 3 GalNAc α	Gal β 1, 3 GalNAc α
17,18	GlcNAc β 1, 6 GalNAc α	**Gal β 1,4** GlcNAc β 1, 6 GalNAc α
19,20	GlcA α	GlcA α
21,22	Fuc α	Fuc α
23,24	Fuc β	Fuc β

Figure 1

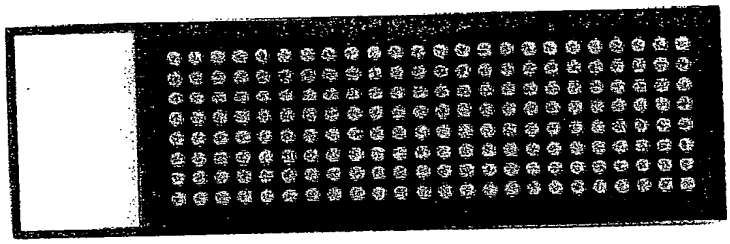


Figure2

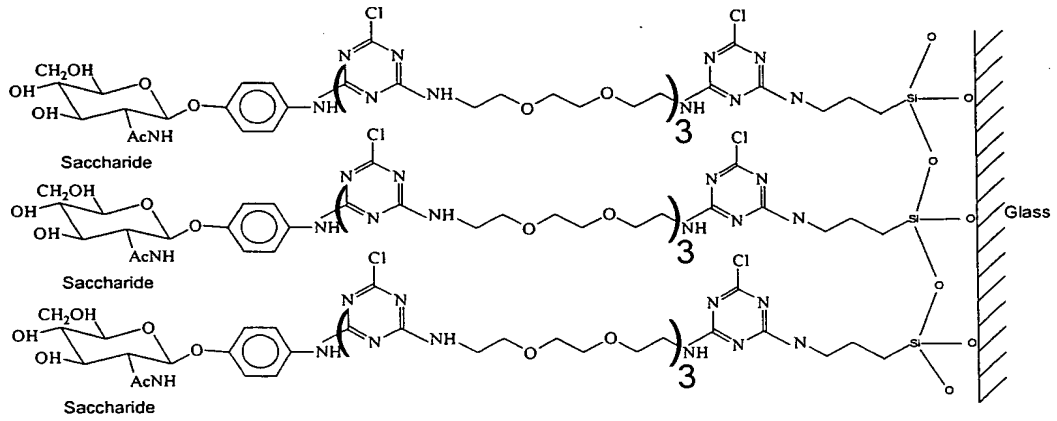


Figure 3

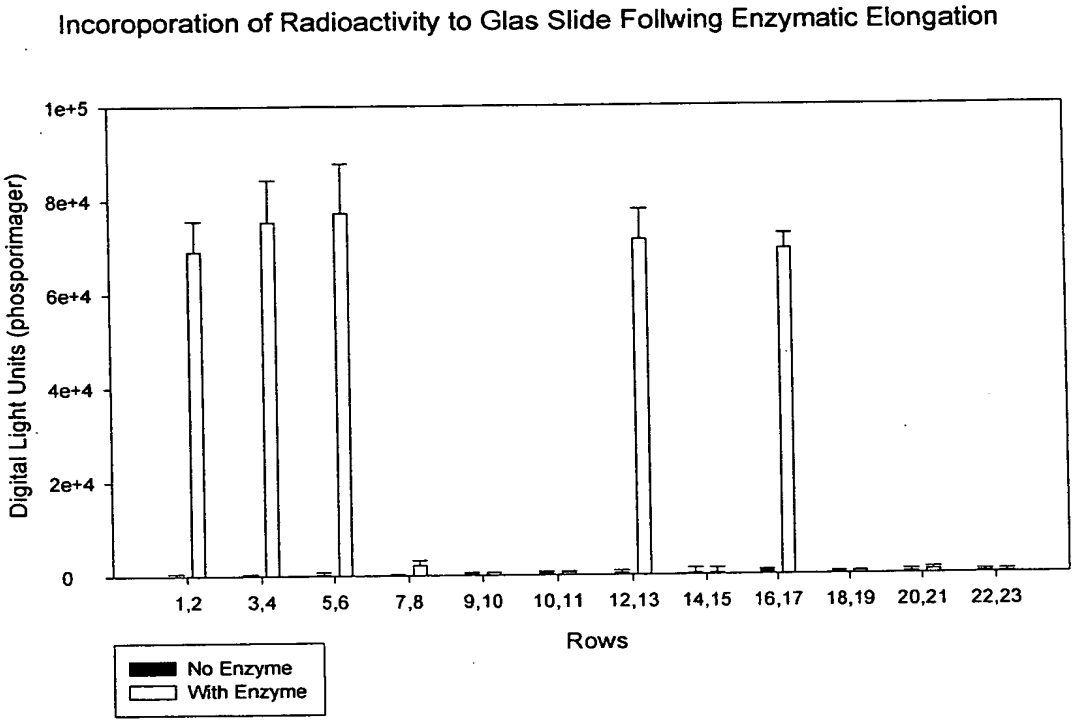

Incoroporation of Radioactivity to Glas Slide Follwing Enzymatic Elongation
Digital Light Units (phosporimager)
1e+5
8e+4
6e+4
4e+4
2e+4
0
1,2
3,4
5,6
7,8
9,10
10,11
12,13
14,15
16,17
18,19
20,21
22,23
Rows
No Enzyme
With Enzyme

Figure 4

Radiograph of the slide after enzymatic reaction, the red lines define the wells area.